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The Influence of Bacteriocins on Resistance to Infection by Gram-negative Bacteria. I. The Effect of Colicin on Bactericidal Power of Blood *

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The gram-negative bacteria in the bowel, urine, and tissues in healthy and infected subjects elaborate powerful antibacterial substances known as colicins. Colicins kill gram-negative bacilli of the same, or closely related, species *in vitro* and have been examined for antibacterial action *in vivo*. Halbert and Swick (1) found that antibacterial substances, similar to colicins, were elaborated in the tissues of mice with peritoneal and subcutaneous infections produced by *Escherichia coli*. Branche, Young, Robinet, and Massey (2) obtained evidence that the ability of some *E. coli* strains to maintain themselves in the human intestine is associated with the production of colicins that suppress competing strains. In order to obtain more definite information on the activity of colicins *in vivo*, it would be advantageous to administer these substances to animals and examine their effect on the antibacterial properties of the body fluids. Until now, such experiments have been limited by the toxicity that results from an intimate association of colicins with the somatic O antigen, or endotoxin. In the present studies, an attempt was made to separate the bactericidal substance from the toxic O antigen, so that the colicin could be inoculated into mice and its effect observed on the bactericidal power of their sera. Mice were chosen for this purpose because their sera normally lack bactericidal power (3).

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Methods

Bacterial strains. A strain of *E. coli* CFl was obtained from N. G. Heatley and H. W. Florey for producing colicin. The *E. coli* strain CFl used in this work had been examined by A. Gratia, who found it identical to his *E. coli* V. In most experiments the antibacterial activity of the colicin was tested against a susceptible strain known as *E. coli* W1895. This strain is a universal indicator of colicin activity and was obtained from Charles Brinton. In a few experiments *E. coli* O:113 was also used as a susceptible strain; it was originally isolated from the blood and urine of a patient and has been thoroughly examined in this laboratory and elsewhere for the production of endotoxin and its behavior under various experimental conditions (4, 5).

Production of colicin V. Colicin was obtained by a modification of the methods of Gratia (6) and Heatley and Florey (7). Gratia observed that colicin passed freely into cellophane bags from broth cultures of *E. coli* V and was recovered in the sterile fluid within the bag. Since the aim of our study was to separate colicin from bacterial endotoxin, this technique was selected because it would allow colicin to enter the cellophane bag and exclude the nondialyzable endotoxin macromolecule. In order to avoid contaminating pyrogens or endotoxins in the culture fluid, a culture medium was devised for most experiments from materials containing no detectable endotoxin or pyrogens. The basic ingredient was a 5% solution of amino acids and small peptides known commercially as Aminosol 5%, and prepared by partial acid hydrolysis of fibrin at the Abbott Laboratories for intravenous administration to patients. Each liter of 5% Aminosol solution was fortified with 5.0 g glucose and 0.5 g thiamin. In a few experiments nonpyrogenic lots of trypticase soy broth also proved suitable for preparing nonpyrogenic colicin V.

Cellophane dialysis tubing, with a diameter of 1⅛ inches, was filled with 10 ml phosphate buffer pH 8 (Na_2HPO_4 M/5, 9.4 ml–$NaH_2PO_4H_2O$ M/5, 0.6 ml) and 1 mg nicotinic acid. The buffer-filled dialysis tubing was tied securely and placed in a 100-ml beaker so that one end was folded over its rim. Five ml of the Aminosol culture medium was added to the beaker, the top covered with aluminum foil, and the whole unit autoclaved at 120° C for 10 minutes. One drop of an 18-hour trypticase soy broth culture of *E. coli* CFl, the colicin producer,

was dropped on top of the sterile tubing by carefully lifting the lid of aluminum foil and incubating for 36 to 40 hours at 37° C. The colicin was harvested by aseptically removing the sterile fluid from within the cellophane bag. Sterility was checked on blood agar and in trypticase soy broth by inoculating them with 0.1-ml portions of the colicin solution. Contaminated solutions were discarded. Sterile colicin preparations were stored up to 30 days at 4 to 5° C or indefinitely after lyophilization at room temperature in a desiccator containing $CaCl_2$. Lyophilized colicin was reconstituted with water before use. The titer of colicin activity was determined by making serial twofold dilutions in water, spotting it on trypticase soy agar plates with a standard wire loop, and overlaying with soft (0.7%) agar containing 0.1 ml of a 6-hour trypticase soy broth culture of the test organism *E. coli* W1895. The plates were incubated at 37° C for 18 hours, and the highest dilution producing a circular zone of bacterial inhibition was noted.

Results

General properties of colicin V[1]

The titer of activity against *E. coli* W1895 ranged from 1:32 to 1:128. This activity was increased by replacing less water than that removed by lyophilization. The pH was 8.2 on removal from the cellophane sac, and colicin activity was unchanged after neutralization to pH 7.0 with 1 N HCl. Colicin activity was retained after autoclaving at 121° C for 30 minutes, but the titer fell 16-fold. The undiluted colicin, when spotted on agar, inhibited 35% ($\frac{5}{14}$) strains of *E. coli* isolated from human urine.

Although colicin V was recovered from within cellophane dialysis tubing after bacterial growth on its external surface, the product so obtained did not readily pass out through dialysis tubing. When colicin V was transferred from within the original tubing to fresh cellophane tubing ($\frac{3}{4}$ inch wide), and dialyzed against sterile Aminosol solution for 4 hours, its titer dropped only 4-fold. At the same time, Na concentration fell from 350 mEq per L to 5 mEq per L. No significant colicin activity was lost upon dialysis for 1 hour against sodium phosphate buffer (pH 7).

Crystalline trypsin, in a concentration of 0.5 mg per ml of colicin solution, completely inactivated the colicin V after incubation at 37° C for 25 minutes so that no bacterial inhibition was noted when the undiluted colicin solution was spotted to agar.

Assay of colicin for the presence of somatic antigen (endotoxin) of E. coli CFl

Tests for somatic antigen were based on its two principal properties: toxicity and antigenicity. The most sensitive test of toxicity is pyrogenicity (8). The most direct test of the antigenic relationship between colicin and somatic antigen is neutralization of colicin activity by antisera against the corresponding somatic antigen (9).

a) Pyrogen assay. Six albino rabbits housed in air conditioned quarters and weighing 2.5 to 3.0 kg were conditioned so that their temperatures did not fluctuate more than 0.5° F. One ml of the solution of colicin was slowly injected into the ear vein of each of the six rabbits. Temperatures were taken rectally with a thermistor apparatus (Sargent) just before injection, 30 minutes later, and hourly thereafter for 6 hours.

None of the six rabbits inoculated with 1.0 ml colicin V developed fever during the 6-hour period of observation. The minimal pyrogenic dose of *E. coli* endotoxin in this laboratory is 0.00045 μg.

b) Colicin activity in the presence of antibody to somatic antigen. Antisera were prepared by three intraperitoneal injections of 0.05 mg *E. coli* CFl somatic antigen at weekly intervals in 36 Swiss mice (CFl), 10 weeks of age. The Boivin somatic antigen was prepared by a standard method in this laboratory (10). Blood was obtained in capillary Pasteur pipettes from the retro-orbital plexus of each immunized mouse before immunization and 9 days after the last injection of somatic antigen. The blood samples before immunization were collected in 4 pools and the ability to precipitate somatic antigen compared with 4 corresponding pools of mouse sera obtained after immunization. Precipitin tests were done in capillary glass tubes by drawing up serum and a 0.1% solution of somatic antigen in 0.9% NaCl solution. The somatic antigen was precipitated at 37° C by pooled sera from immunized mice, but not by the sera obtained before immunization.

The antibacterial activity of colicin V in the presence of antibody to somatic antigen was

[1] All observations recorded in this paper refer to sterile colicin V obtained from within the cellophane sac by the technique described under Methods.

examined by a bactericidal test with *E. coli* W1895, the susceptible indicator strain. The solution of sterile colicin with a titer of 1:64, obtained from within the dialysis tubing, was diluted 1:4 with 5% Aminosol and 0.5 ml of the colicin solution added to 0.5 ml pooled immune mouse sera. Control tests were performed by adding 0.5 ml colicin to 0.5 ml pooled mouse sera obtained before immunization; 0.5 ml Aminosol to 0.5 ml pooled immune mouse sera; and 0.5 ml Aminosol to 0.5 ml pooled sera obtained before immunization. A 6-hour trypticase soy broth culture of *E. coli* W1895 was diluted 10^{-4} in trypticase soy broth and 0.1 ml added to 0.9 ml of each mixture of colicin and serum, and to each mixture of Aminosol and serum. In addition 0.1 ml of the broth culture was added to 0.9 ml serum alone. Plate counts of viable bacteria were made with 0.1-ml portions removed after 0, 1, 6, and 24 hours incubation at 37° C.

Mouse antiserum that precipitated somatic antigen of *E. coli* CF1 (the bacterial strain producing colicin V) did not neutralize the bactericidal activity of colicin V (Table I). A 1:8 dilution of colicin V in nonimmune serum and in O antiserum sterilized an inoculum of approximately 10^3 bacteria per ml within 6 hours.

TABLE I

Failure of precipitating antiserum against somatic antigen from the colicinogenic strain of Escherichia coli CF1 to neutralize the bactericidal action of CF1 colicin V

Group of mice providing serum-pool	Serum obtained before or after immunization with somatic antigen of colicinogenic *E. coli* CF1	Number of viable *E. coli* W1895 per ml of serum at each hour of incubation at 37° C											
		Serum + Colicin				Serum + Aminosol				Serum alone			
		0 hr	1 hr	6 hr	24 hr	0 hr	1 hr	6 hr	24 hr	0 hr	1 hr	6 hr	24 hr
I	Before	990	7	0	0	1,360	2,200	22,000	Innum.*	1,260	3,200	Innum.	Innum.
	After	900	5	1	0	1,630	1,500	16,200	Innum.	1,320	3,130	Innum.	Innum.
II	Before	1,040	10	0	0	1,800	2,100	Innum.	Innum.	1,350	2,800	Innum.	Innum.
	After	930	22	0	0	1,860	1,900	Innum.	Innum.	1,600	7,800	Innum.	Innum.
III	Before	1,010	12	0	1,450	1,840	2,630	Innum.	Innum.	1,200	2,900	Innum.	Innum.
	After	1,700	7	0	0	1,760	2,560	Innum.	Innum.	1,300	2,420	24,220	Innum.
IV	Before	1,520	11	1	0	2,240	2,840	Innum.	Innum.	1,000	1,700	34,000	Innum.
	After	1,750	3	0	0	1,900	2,900	Innum.	Innum.	1,400	2,520	Innum.	Innum.

* Innumerable.

Bactericidal power of mouse blood and serum after subcutaneous inoculation of colicin V.

Twelve white Swiss mice, weighing 26 to 33 g, and obtained from Carworth Farms (CF1), were inoculated subcutaneously as follows:

1) Each of six mice received in the buttocks 1.1 ml lyophilized colicin V redissolved in water to give a titer of 1:32. The solution was isotonic (332 mOsm per L) with body fluids as measured in the Fiske osmometer. Approximately 0.8 ml of blood was drawn from the heart of each mouse 1 day before inoculation of colicin. Three mice were exsanguinated by cardiac puncture at 30 minutes and the remaining three at 60 minutes after the injection of colicin. After the bloods had clotted and retracted at room temperature, the sera were separated by centrifugation and examined simultaneously for bactericidal activity.

2) Six mice received 1.0 ml of nonlyophilized colicin V (titer 1:32) in each buttocks. One-

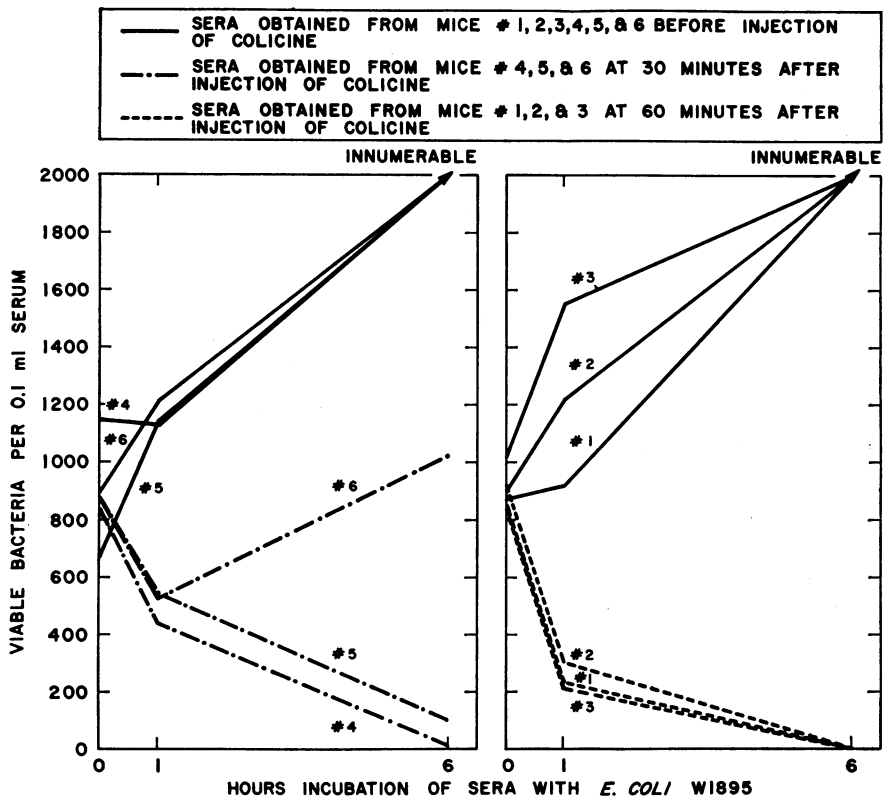

FIG. 1. BACTERICIDAL ACTION OF MOUSE SERA AGAINST *E. coli* W1895 AFTER SUBCUTANEOUS INOCULATION OF COLICIN V.

half ml blood was obtained from the retro-orbital plexus of each mouse with a capillary Pasteur pipette that had been rinsed with sterile heparin. Blood samples were collected just before injection of colicin and 30, 60, and 120 minutes after the injection of colicin. The heparinized bloods were examined immediately for bactericidal activity. The object of this experiment was to perform serial determinations of bactericidal power on the whole blood of individual mice.

The bactericidal test was done with a 10^{-4} dilution of a 7-hour trypticase soy broth culture of *E. coli* W1895. An inoculum of 0.05 ml of bacterial suspension was added to 0.5 ml serum or 0.2 ml of whole blood, and plate counts of viable bacteria were made with 0.05 ml at 0, 1, 5 to 6, and 24 hours after incubation at 37° C.

Sera obtained by cardiac puncture from mice at 30 and 60 minutes after subcutaneous inoculation of colicin V became strongly bactericidal and killed approximately 10,000 organisms per ml in 6 hours (Figure 1). Similar bactericidal activity was observed at the same time intervals in the whole blood of mice obtained by repeated bleeding from the retro-orbital plexus (Figure 2). The bactericidal activity of the whole blood was present at 2 hours after injection of colicin V but disappeared at 24 hours. Mouse bloods and sera obtained before injection of colicin possessed no bactericidal power and allowed heavy multiplication of *E. coli* W1895.

Effect of endotoxin on colicin activity in the presence and absence of serum

Lyophilized colicin was reconstituted in sterile distilled water to give a titer of 1:64, and dialyzed for 1 hour against phosphate buffer (pH 7.0). Mouse sera were pooled from bloods obtained by cardiac puncture. Human serum was taken from fresh cord blood in order to avoid the 17–21 S class of antibody to the *E.* coli endotoxin (11). The cord serum was heated 30 min-

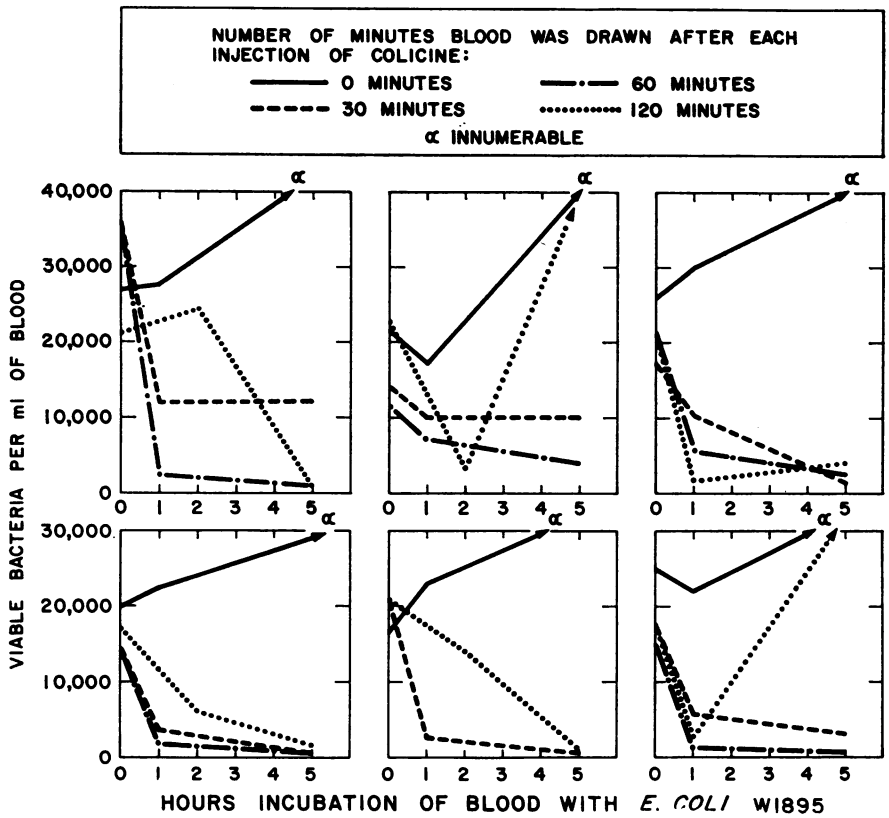

FIG. 2. BACTERICIDAL ACTION OF WHOLE BLOOD FROM SIX MICE INOCULATED SUBCUTANEOUSLY WITH COLICIN V. The group of bacterial survival curves in each box represents serial determinations in one of the six mice.

utes at 56° C to inactivate its bactericidal action; mouse serum was not inactivated in this way because it lacked bactericidal power for *E. coli.* To 0.5 ml of colicin solution was added 0.5 ml of serum or 0.9% NaCl solution. To 0.9 ml of the colicin-serum or colicin-saline mixtures was added 0.1 ml of a solution of either *E. coli* O: 113 endotoxin or *Salmonella abortus equi* endotoxin to give final concentrations indicated in Tables II and III. The strain of *S. abortus equi,* from which the endotoxin was extracted, was resistant to the colicin, whereas the strain of *E. coli* O: 113 was susceptible. The Boivin type endotoxins of *E. coli* O: 113 and *S. abortus equi* were prepared by the same technique. The mixtures of colicin and endotoxin, or colicin-serum and endotoxin, as well as the controls (serum alone, colicin alone), were incubated for 2 hours at 37° C. Then each was inoculated with 0.1 ml of a 10^{-4} dilution of a 5-hour trypticase soy broth culture of *E. coli* W1895. The mixtures were incubated further at 37° C and pour plates made for counts of viable bacteria at 0, 1, and 6 hours, as specified in Table III.

The results in Table II show that endotoxins of either *E. coli* O: 113 or *S. abortus equi,* in concentrations of 0.1 mg per ml, inhibited the bactericidal effect of colicin V against *E. coli* W1895. This inhibitory effect of endotoxin was not observed in normal mouse serum or in serum from human cord blood (Table III). In a concentration of 0.01 mg per ml or less there was no inhibition of colicin activity by either of the two endotoxins. These results show that colicin V can be inhibited by endotoxins from bacteria that are either susceptible (*E. coli* O: 113) or resistant (*S. abortus equi*) to the colicin.

Effect of serum on the action of colicin

The colicin solution was mixed with equal amounts of pooled mouse serum, and twofold

TABLE II

Inhibition of colicin activity by endotoxin in 0.9% NaCl solution but not in pooled mouse serum

Species of endotoxin	Concentrations of endotoxin	Pooled mouse serum	Colicin V	Number of viable *E. coli* W1895 per 0.1 ml at each hour of incubation at 37° C		
				0 hr	1 hr	6 hr
	mg/ml					
E. coli O:113	0.1	0	+	400	270	8,200
	0.1	+	+	520	52	18
	0.01	0	+	430	110	110
	0.01	+	+	520	36	5
	0.001	0	+	330	75	110
	0.001	+	+	500	35	10
Salmonella abortus equi	0.1	0	+	360	310	25,900
	0.1	+	+	580	50	27
	0.01	0	+	530	80	90
	0.01	+	+	550	82	10
	0.001	0	+	440	80	90
	0.001	+	+	510	60	20
None		0	+	510	51	300
		+	+	450	35	120
		+	0	420	760	11,700

serial dilutions were made with the pooled mouse serum as the diluent. The colicin solution was also diluted serially in 0.9% NaCl solution. To 0.7-ml portions of each of the serial dilutions, was added 0.1 ml of the 10^{-4} dilution of the 8-hour culture of *E. coli* W1895. Serum alone was similarly inoculated, and incubated at 37° C, and viable bacterial counts were made with pour plates at 0, 1, and 6 hours, as specified in Table IV. The experiment was then repeated with inactivated human cord serum (Table V).

The effect of heat inactivation on the bactericidal power of serum in the presence and absence of colicin was examined with normal rabbit serum that, unlike mouse serum, killed *E. coli* W1895. Ten ml of serum from a normal rabbit was divided into two equal portions. One portion was mixed with an equal volume of colicin solution and the other with an equal volume of 0.9 NaCl solution. Colicin solution was similarly mixed with an equal portion of 0.9% NaCl solution. One-half of each of the colicin-serum, the serum-saline, and the colicin-saline mixtures was heated at 56° C for 30 minutes. Then 1.0 ml of each of the six samples was inoculated with 0.1 ml of a 10^{-4} dilution of a 5-hour culture of *E. coli* W1895; another 1.0 ml of each sample was similarly inoculated with *E. coli* O:113. The inoculated mixtures were incubated at 37° C, and 0.1 ml was removed for plate counts of viable bacteria at the time intervals specified in Figure 3.

TABLE III

Inhibition of colicin activity by E. coli O:113 endotoxin in 0.9% sodium chloride solution, but not in human cord serum

Concentration of endotoxin	Human cord serum inactivated 56° C	Colicin V	Number of viable *E. coli* W1895 per 0.1 ml at each time of incubation at 37° C		
			0 hr	1 hr	6 hr
mg/ml					
0.1	0	+	550	370	7,000
0.1	+	+	490	72	15
0	0	+	480	145	18
0	+	+	488	80	0
0	+	0	650	700	3,600

Pooled mouse serum, rabbit serum, and serum from human cord blood increased the bactericidal effect of colicin V, as shown in Tables III, IV, and V, and Figure 3. Figure 3 shows that rabbit serum containing colicin V remained bactericidal after heating at 56° C for 30 minutes, a manipulation that destroyed the bactericidal effect of serum alone through the inactivation of comple-

TABLE IV

Effect of pooled mouse serum on colicin V activity

	Reciprocal colicin dilution	Serum	Number of viable *E. coli* W1895 per 0.1 ml at each hour of incubation		
			0 hr	1 hr	6 hr
Mouse serum Experiment I	2	0	590	10	30
		+	490	160	0
	4	0	615	220	46
		+	670	370	10
	8	0	710	260	210
		+	670	95	84
	16	0	706	140	560
		+	700	127	28
	32	0	680	310	440
		+	660	132	75
	No colicin	+	800	610	Innumerable (approx. 60,000)
Mouse serum Experiment II	2	0	350	52	260
		+	360	76	*
	4	0	340	31	2,330
		+	330	50	290
	8	0	380	160	4,680
		+	300	50	14
	16	0	370	260	30,300
		+	350	80	40
	32	0	320	300	33,000
		+	320	110	110
	64	0	280	350	46,500
		+	370	140	850
	128	0	290	370	Innumerable
		+	300	200	3,200
	256	0	340	370	Innumerable
		+	360	300	6,000
	No colicin	+	360	540	9,000

* No data.

ment. Serum plus colicin, after such heat treatment, sterilized in 24 hours an inoculum of *E. coli* O:113 that was only partially inhibited by colicin V in the absence of serum, illustrating again the potentiating effect of serum on colicin V. The results show that the bactericidal action of serum containing colicin cannot be reduced by inactivation at 56° C for 30 minutes.

Reproducibility of results

All experiments were repeated at least three times and the results confirmed each time.

Discussion

Although colicin V can be produced within cellophane sacs from bacteria growing on their exterior, the final products cannot, for the most part, be dialyzed. This surprising discrepancy was first pointed out by Gardner (12), but her observation apparently did not come to the attention of Hutton and Goebel (13) during their studies on the association between colicins and the toxic somatic antigens, or endotoxins. They prepared colicins by precipitation with ethanol from culture supernates and found the colicins non-

TABLE V

Effect of human cord serum (inactivated 56° C) on colicin V activity

Reciprocal colicin dilution	Serum	Number viable *E. coli* W1895 per 0.1 ml at each hour of incubation			
		0 hr	1 hr	6 hr	24 hr
2	0	490	51	160	1,960
	+	540	26	0	0
4	0	440	120	4,500	Innumerable
	+	560	107	3	0
8	0	480	200	14,700	Innumerable
	+	590	241	16	0
16	0	540	500	Innumerable	Innumerable
	+	580	370	11	0
32	0	520	640	Innumerable	Innumerable
	+	510	570	40	7
64	0	500	1,360	Innumerable	Innumerable
	+	580	680	8,760	28,800
128	0	520	1,030	Innumerable	Innumerable
	+	600	860	12,000	36,800
256	0	540	1,400	Innumerable	Innumerable
	+	500	910	9,800	18,300
No colicin	+	600	1,100	10,200	21,600

dialyzable and inseparable from the endotoxin. The present experiments were undertaken on the premise that production of colicin within cellophane sacs would exclude the large nondialyzable macromolecules of endotoxin (14). The preparations obtained in this fashion showed neither the toxic nor immunologic properties of the somatic antigen obtained from the colicinogenic strain of *E. coli*. Assays for pyrogenicity and for neutralization of colicin activity by O antibody gave convincing evidence that the colicin had been separated by the cellophane from the large endotoxin molecules. The smaller yield of colicin within the sac than that obtained in the supernate of ordinary broth cultures may reflect a strong affinity of colicin for endotoxin.

Upon separation from endotoxin, the properties of colicin can be observed without the complicating effects of endotoxin. Much of the endotoxin injected into the skin appears to be bound locally, and the remainder is rapidly removed from the blood by the reticuloendothelial system (4). This behavior of endotoxin could influence the circulation of colicin through their intimate association. Endotoxin also produces severe vascular disturbances and renal injury that could affect the concentration of colicin in the blood, its clearance by the kidneys, and its biochemical environment in the body fluids. In the absence of endotoxin, colicin activity was found in the circulation as long as 2 hours after subcutaneous injection and gave the blood strong bactericidal powers. In order to demonstrate this effect, mice were chosen as test subjects because they do not possess the serum bactericidal power that most other animals exhibit against gram-negative bacteria. The heat stable property of colicin, however, made it possible to show that this substance also exerts its bactericidal effect in sera that are normally bactericidal unless complement is inactivated by heat. The bactericidal power of rabbit serum containing colicin did not change after heating and suggests that colicin might be one of the factors contributing to the bactericidal power that sometimes remains after complement is inactivated (15, 16).

In addition to its resistance to heat in serum, colicin also appeared to resist the proteolytic enzymes in serum and blood. Because of its susceptibility to proteolytic enzymes, it has been suggested that colicin would be destroyed in the bowel so that it could not influence the fecal

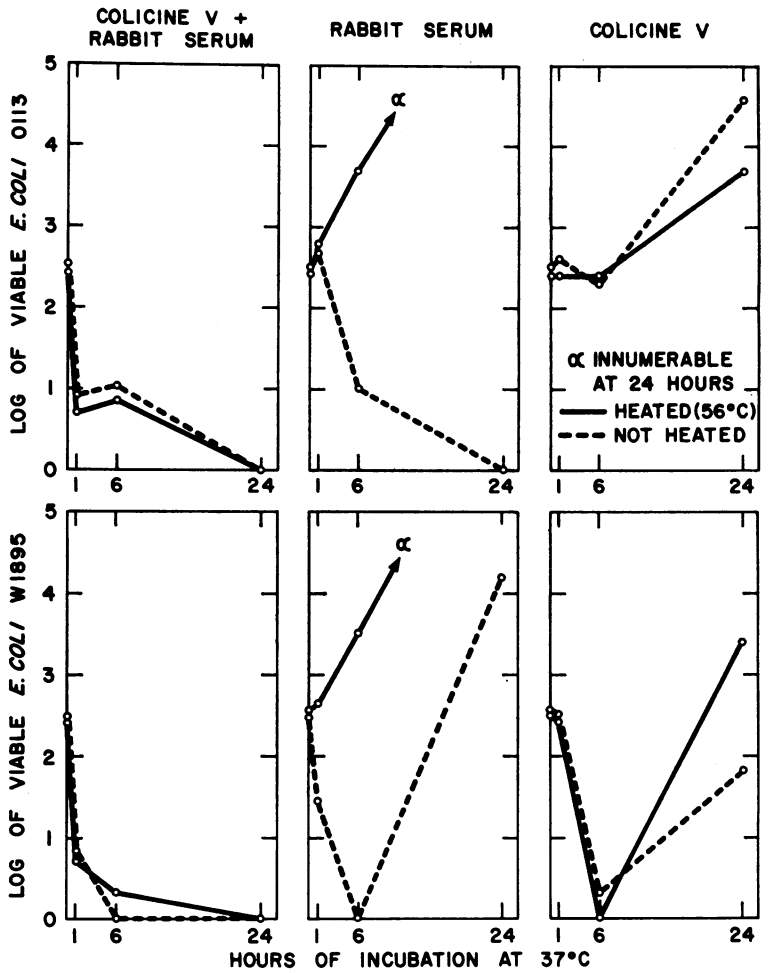

FIG. 3. EFFECT OF COLICIN ON THE RESISTANCE OF SERUM-BACTERICIDAL PROPERTY TO HEAT. Heat destroys bactericidal action of rabbit serum unless colicin is present.

flora. The same inactivation by proteolysis has been anticipated in other body fluids and led to the prediction that colicins would be ineffective in them. The present studies show, on the contrary, that colicins can remain active after circulating in the body fluids for at least 2 hours, and that serum both augments and protects the action of colicins.

The augmentation of colicin activity by serum has been noted previously in studies of bacteriostasis (7, 12). The present study demonstrates that its bactericidal activity is also increased in serum, including that of mice, rabbits, and human subjects. In order to account for this enhancement of colicin activity by serum, a study was made to determine whether or not the lysozyme in serum might be involved in the bactericidal action of colicin. Lysozyme is said to be involved in the bactericidal activity of complement and antibody (17), but we could not obtain conclusive evidence that lysozyme enhanced colicin activity (18). A more promising lead came from our observation that colicin was inactivated by endotoxin in 0.9% NaCl solution, but not in serum. Such protection of colicin by serum from the inactivating effect of endotoxin would maintain colicin activity at the bacterial cell wall, where high concentrations of endotoxin might otherwise reduce its potency. In feces, on the other hand, serum could not protect colicin from the heavy

concentrations of endotoxin, both in and out of the bacterial cells, and colicin inactivation would be greater. In fact the combined action of endotoxin and proteolytic enzymes in the bowel might account for the failure of colicin-producing bacteria to affect the fecal flora of animals (19).

The mechanism of colicin inactivation by endotoxin is unknown. The neutralization of bacteriophage by the lipopolysaccharide antigens of phage-susceptible bacteria is thought to result from specific combination between phage and specific receptor substances (20). Neutralization of colicins by endotoxin could not develop from a reaction between colicin and specific receptors because it occurs with the endotoxins of both susceptible and resistant bacteria. The mechanism by which serum protects colicin from inactivation by endotoxin is also unknown. At least two serum factors are known to react with endotoxin and might affect its ability to neutralize colicin: one of these is "natural" antibody, and the other EDTC—the endotoxin detoxifying component of Skarnes, Rosen, Shear, and Landy (21). The failure of endotoxin to reduce colicin activity in serum from human cord blood eliminates natural antibody from consideration because the 17–21 S class of gamma globulins does not traverse the placenta (10). It is also unlikely that EDTC inhibits the action of endotoxin on colicin because EDTC functions only when citrate is present to reduce the concentration of ionized calcium. It is possible that the protection of colicin from endotoxin by serum is related to the enhancement of colicin activity by serum and that this occurs through a mechanism which removes the colicin from exposure to endotoxin.

The strong bactericidal activity of small amounts of colicin in serum observed in these experiments might also occur under natural conditions through absorption of colicin from the bowel. Absorption from the bowel was considered unlikely until now because colicin was thought to be inseparably bound to endotoxin. The finding that during its formation colicin can pass through a membrane independently of endotoxin brings to mind the intriguing possibility that colicins pass similarly into the intestinal capillaries before they are inactivated by proteolytic enzymes of the bowel. Absorption of colicin might also occur in this way from organs such as the urinary bladder where infection with colicin-producing bacteria is frequent. At least 30% of the coliform bacteria that we have isolated from the urine of patients have been found to produce colicins against a single indicator strain, and the rate rose with the use of more indicators. The elaboration of colicin in the urine and its effect on experimental infection is described in the second paper of this series (22).

Summary

The intimate association of colicin with endotoxin has interfered with the study of colicin *in vivo*. In order to examine the properties of colicin *in vivo* without the complicating effects of endotoxin, an attempt was made to separate them by growing *E. coli* CF1 on the outside of cellophane dialysis tubing. Colicin V passed into the bag, but not out. The colicin in the cellophane bag was sterile, relatively heat resistant, active against *E. coli* strains in high dilution, and produced no fever or other signs of endotoxin activity in rabbits. Thirty to 120 minutes after subcutaneous inoculation of this nontoxic colicin, mouse blood and serum, which normally contain no bactericidal activity, became strongly bactericidal for the universal indicator *E. coli* W1895. Mixture of serum with colicin V *in vitro* greatly increased the bactericidal titer of the colicin and abolished the inhibitory effect of endotoxins on colicin. Antisera to the endotoxin of *E. coli* CF1 (from which colicin V was prepared) did not reduce the colicin V bactericidal activity.

These findings suggest that colicin V can be separated from the toxic and antigenic fractions of endotoxin, and can contribute to the heat-resistant bactericidal property of normal blood. The possibility that colicin from intestinal bacteria can diffuse through the bowel wall into the blood stream is discussed.

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